

AWARD NUMBER: W81XWH-14-1-0221

TITLE: Targeting the Mevalonate Pathway to Reduce Mortality from Ovarian Cancer

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE: October 2015		2. REPORT TYPE: Annual		3. DATES COVERED: 30 Sep 2014 - 29 Sep 2015	
4. TITLE AND SUBTITLE:  Targeting the Mevalonate Pathway to Reduce Mortality from Ovarian Cancer				5a. CONTRACT NUMBER W81XWH-14-1-0221	
				5b. GRANT NUMBER W81XWH-14-1-0221	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S): Kala Visvanathan				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  The Johns Hopkins University Johns Hopkins Bloomberg School of Public Health Epidemiology Dept., E6141 615 N. Wolfe Street Baltimore, MD 21205				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The primary purpose is to evaluate whether statins, a well-known cholesterol-lowering agent, will improve survival in women with epithelial ovarian cancer. Aim 1: a) prospectively examine whether statin use reduces both cancer specific and overall mortality among approximately 7886 women with epithelial ovarian cancer after adjustment for stage, grade, treatment, histologic subtype, co-medication use, type of surgery and type of hospital. Statin use will be compared to non-users as well as users of other lipid lowering agents; and b) test whether the association is modified by: i) dose and duration, ii) timing of the intervention (pre-diagnosis versus post diagnosis use), iii) histologic subtype and iv) degree of adherence. Aim 2:a) assess the anti-tumor effect of lovastatin, (a commonly prescribed statin) alone or in combination with carboplatin/paclitaxel in a mouse orthotopic tumor xenograft model bearing luciferase-expressing OVCAR3, SKOV3, and A2780 cells and; b) determine the molecular mechanism by which lovastatin inhibits tumor growth. The clinical and translational impact of this project are substantial because unlike other potential new drug treatments, statins are already in wide use, have been shown to have very low toxicity and could therefore be put quickly into practice (if clinical trials confirm their efficacy) and at low cost.					
15. SUBJECT TERMS: cancer mortality, cholesterol-lowering drugs, disease progression, epithelial ovarian cancer, lovastatin, Mevalonate Pathway, prescription drugs, statins, survival, transgenic mouse tumor model					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	23	19b. TELEPHONE NUMBER (include area code)

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**Section I – INTRODUCTION:**

This project is evaluating whether the addition of statins, a well-known cholesterol-lowering agent, will improve survival in women with epithelial ovarian cancer. We hypothesize that both pre-diagnostic and post diagnostic statin use will improve survival among women with epithelial ovarian cancer and that a higher dose or longer duration of use will be associated with a greater reduction in overall mortality. We also hypothesize that the anti-tumor effects of statin is mediated by a pathway that included enzymes that are involved in farnesylation. To test our hypothesis, we proposed a multidisciplinary approach that includes conducting both a large epidemiological study and a number of preclinical studies.

**Section II – KEYWORDS:**

Bisphosphonates  
 Cancer mortality  
 Carboplatin/paclitaxel  
 Cholesterol-lowering drugs  
 Disease progression  
 Drug exposure  
 Epithelial Ovarian Cancer  
 Finnish Cancer Registry  
 Lovastatin  
 Mevalonate pathway  
 Prescription drugs  
 Social Insurance Institute of Finland  
 Statins  
 Survival  
 Transgenic mouse tumor model

**Section III – ACCOMPLISHMENTS:****A. What were the major goals of the project?**

<b>SPECIFIC AIM 1:</b>	<b>Timeline</b>	<b>Status</b>
1a) examine whether statin use, reduces both cancer specific and overall mortality among approximately 7886 women with epithelial ovarian cancer; 1b) test whether the association is modified by dose and duration, timing of the intervention (pre vs. post diagnosis use), histologic subtype, and patterns of adherence after adjusting for prespecified covariates.	<b>Start date</b> <b>9/30/14</b>	
<b>Major Task 1:</b> Obtain approvals from Johns Hopkins SPH IRB, DoD HRPO, MTA and USAMRAA.	Months	<b>Completed</b>
Subtask 1: Protocol development and submission to Johns Hopkins SPH IRB for review and approval.	0-2	<b>Completed</b> JH SPH IRB Determination
Subtask 2: Submission of Johns Hopkins IRB approval and related material for DoD's HRPO review and approval.	0-2	<b>Completed</b> DoD HRPO acknowledgement
Subtask 3: Develop MTA- Johns Hopkins & Tampere University, Finland.	0-3	<b>Completed</b> MTA fully executed 1/6/15

<b>Major Task 2:</b> Request for data variables to be studied.		<b>Completed</b>
Subtask 1: Submit proposal that includes a specific data request to Finnish Cancer Registry so they can identify variables.	0	
1. Proposal approval and data request completed.	4-5	<b>Completed</b>
<b>Major Task 3:</b> Clean data, generate study specific variables and analyze dataset on statin use and mortality among OV CA cases as well as associations/ modifications factors such as dose, duration, pre/post-diagnosis use, histologic subtype and patterns of adherence.		<b>In progress</b>
Subtask 1: Data preparation which includes cleaning, extracting medication use from prescription records, recoding data, generating new study specific variables.	4-9	<b>In progress</b>
Subtask 2: Conduct primary data analysis regarding stains and mortality among OV CA cases & interpret results (aim 1a).	9-13	<b>Pending</b>
Subtask 3: Conduct secondary data analysis regarding associations or modification of statin use & adherence and a number of sensitivity analyses (aim 1b).	14-20	<b>Pending</b>
Subtask 4: Prepare and submit an abstract to a national professional meeting (either AACR or ASCO).	14-20	<b>Pending</b>
Subtask 4: Prepare and submit manuscript for journal submission and begin developing subsequent study based on results.	16-24	<b>Pending</b>
1. Main data analysis completed.	14-16	<b>Pending</b>
2. Secondary analysis completed.	14-20	<b>Pending</b>
3. Presentation of data at national professional meeting (either AACR or ASCO).	14-20	<b>Pending</b>
4. Journal manuscript submitted (i.e. Cancer Research or JCO).	22-24	<b>Pending</b>
5. Data used in support of applying for further funding to study new agents in the mevalonate pathway.	22-24	<b>Pending</b>
<b>SPECIFIC AIM 2:</b> 2a)access anti-tumor effects of lovastatin alone or in combination with carboplatin/paclitaxel in a mouse orthotopic tumor xenograft model bearing luciferase-expressing OVCAR3, SKOV3, OVCAR5 cells; 2b) determine molecular mechanism by which lovastatin inhibits tumor growth.	<b>Start date</b> <b>9/30/14</b>	
<b>Major Task 1:</b> Obtain approvals from Johns Hopkins Animal Care and Use Committee (IACUC) and the DoD Animal Care and Use Review Office (ACURO).		<b>Completed</b>
Subtask 1: Develop and submit proposal to JH IACUC for review.	0-4	<b>Completed</b>
Subtask 2: Submit documents for DoD ACURO for review.	0-4	<b>Completed</b>
1. Local JH IACUC Approval & DoD ACURO Approval.	2-4	<b>Completed</b>
<b>Major Task 2:</b> In vivo anti-tumor study.		
Subtask 1: Establish ovarian cancer cell lines with luciferase-expressing constructs (OVCAR3, SKOV3, & OVCAR5 cells.) Cell line source: ATCC	0-4	<b>Completed</b>

Subtask 2: Purchase athymic <i>nu/nu</i> nude mice (n=120) and set ready for mouse tumor model. Mouse: purchased from Harlan Laboratories	5,13	In progress
Subtask 3: Perform viable surgery by orthotopic injection ovarian cancer cells into mouse ovarian bursa.	5,13	In progress
Subtask 4: Treat the mice with lovastatin or vehicle control and monitor tumor load using live imaging system.	6-8, 14-16	In progress
Subtask 5: Perform immunohistochemistry to study the expression of proliferation, autophagy, apoptosis markers, as well as other markers identified in our pilot study.	9-12, 17-20	In progress
1. Determine the anti-tumor effect of lovastatin alone or combination with conventional chemotherapeutic agents.	5-20	In progress
2. Identify biomarkers associated with statin treatment in cancer cells.	5-20	In progress
<b>Major Task 3:</b> Conduct gene knockdown and enzyme inhibitor study to determine which subpathway(s) is mainly responsible for the anti-tumor effects of lovastatin.		Completed
Subtask 1: Purchased siRNAs targeting the enzymes belonging to the three subpathways in the mevalonate metabolism.	3-4	Completed
Subtask 2: Purchase small molecule enzyme inhibitors of the mevalonate subpathways.	3-4	Completed
Subtask 3: Perform gene knockdown using siRNA in ovarian cancer cell lines (same as described in Task 2). Determine the effects of lovastatin on cellular proliferation, autophagy, and apoptosis as well as the expression of new markers identified in our pilot study.	4-16	Completed
Subtask 4: Determine the effects of inhibitors on the phenotypes described in Subtask 3 in the same panel of ovarian cancer cell lines. Compare the alterations of phenotypes between 3 and subtask 4.	4-16	Completed
1. Demonstrate the effects of knockdown in those genes regulating mevalonate pathway.	3-16	Completed
2. Demonstrate the effects of enzyme inhibitors for those proteins regulating the mevalonate pathway.	3-16	Pending
3. Demonstrate that mevalonate pathway is essential for cellular survival and growth in ovarian cancer cells.	3-16	Pending
<b>Major Task 4:</b> Final phase: data analysis, summary, and publication.		Pending
Subtask 1: Analyze and summarize data to support or refute our hypothesis.	13-24	Pending
Subtask 2: Prepare manuscript(s) for journal submission.	13-24	Pending
1. Presentation of data in meetings or conferences such as AACR annual meeting and others.	13-24	Pending
2. Publication of data.	13-24	Pending
3. Generating research findings that can be used in support of applying for further funding opportunity.	13-24	Pending

## **B. What was accomplished under these goals?**

### **Aim 1:**

**Aim 1a) examine whether statin use, reduces both cancer specific and overall mortality among approximately 7886 women with epithelial ovarian cancer; 1b) test whether the association is modified by dose and duration, timing of the intervention (pre vs. post diagnosis use), histologic subtype, and patterns of adherence after adjusting for prespecified covariates.**

**Major Task 1:** Obtain approvals from Johns Hopkins SPH IRB, DoD HRPO, MTA and USAMRAA.

**Task 1-1:** Protocol development and submission to Johns Hopkins SPH IRB for review and approval.

**Progress:** A protocol was submitted and reviewed by the Johns Hopkins SPH IRB and determined that the proposed activity described in the protocol involved secondary data analysis of existing de-identified/de-linked, not publicly available datasets, and that we were not involved in the original data collection. As such, the proposed activity did not qualify as human subjects research as defined by DHHS regulations 45 CFR 46.102, and does not require IRB oversight.

**Task 1-2:** Submission of Johns Hopkins IRB approval and related material for DoD's HRPO review and approval.

**Progress:** HRPO reviewed the Johns Hopkins SPH IRB Determination Letter and concurred and sent a "*Research Not Involving Human Subjects Determination Memorandum*" on 5/28/14 stating the project may proceed with no further requirement for review by the HRPO.

**Task 1-3:** Develop MTA- Johns Hopkins & Tampere University, Finland

**Progress:** A fully executed MTA/Data Use Agreement was put in place 1/5/15. Additionally we have a signed Commission Decision C(2010)593 Standard Contractual Clause (processors).

**Major Task 2:** Request for data variables to be studied.

**Task 2-1:** Submit proposal that includes a specific data request to Finnish Cancer Registry so they can identify variables.

**Progress:** We submitted and obtained approval 12/23/14 from the Finland National Institute for Health & Welfare (THL) to obtain data for ovarian cancer cases registered in the Finnish Cancer Registry (FCR) during 1995-2013. Information on the cancer case's children and 1st degree female relatives, in addition to information on emigrations and possible death was requested from the Finnish Population Register for linkage to FCR to obtain information on the 1<sup>st</sup> degree relatives' breast and ovarian cancer diagnoses. This was to be merged with lifetime information on drug purchases and chronic diseases from the Social Insurance Institute (SII) of Finland. Additionally, information on co-morbidity from the hospital discharge (HILMO) will be linked to the data.

**Major Task 3:** Clean data, generate study specific variables and analyze dataset on statin use and mortality among OV CA cases as well as associations/ modifications factors such as dose, duration, pre/post-diagnosis use, histologic subtype and patterns of adherence.

**Task 3-1:** Data preparation which includes cleaning, extracting medication use from prescription records, recoding data, generating new study specific variables.

**Progress:** The data from various national organization are currently being merged together. The data consists of materials combined from the following health care register sources: 1) the Finnish Cancer Registry (information on ovarian cases); 2) the Social Insurance Institution of Finland (information on drug purchases and special reimbursements); 3) Finnish Population Register Center (information on born children and 1<sup>st</sup> level female siblings, emigration and death); and 4) patient register, HILMO (information on co-morbidity).

**Task 3-2:** Conduct primary data analysis regarding stains and mortality among OV CA cases and interpret the results (aim 1a).

**Progress:** Pending

**Task 3-3:** Conduct secondary data analysis regarding associations or modification of statin use & adherence and a number of sensitivity analyses (aim 1b).

**Progress:** Pending

**Task 3-4:** Prepare and submit an abstract to a national professional meeting (either AACR or ASCO).

**Progress:** Pending

**Task 3-5:** Prepare and submit manuscript for journal submission and begin developing subsequent study based on results.

**Progress:** Pending

## **Aim 2:**

**Aim 2 a) To assess the anti-tumor effects of lovastatin alone or in combination with carboplatin/paclitaxel in a mouse orthotopic tumor xenograft model bearing luciferase-expressing OVCAR3, SKOV3 and OVCAR5 cells, b) To determine the molecular mechanism by which lovastatin inhibits tumor growth.**

**Major Task 1:** Obtain approvals from Johns Hopkins Animal Care and Use Committee (IACUC) and the DoD Animal Care and Use Review Office (ACURO).

**Progress:** The approvals to conduct the proposed animal studies have been obtained.

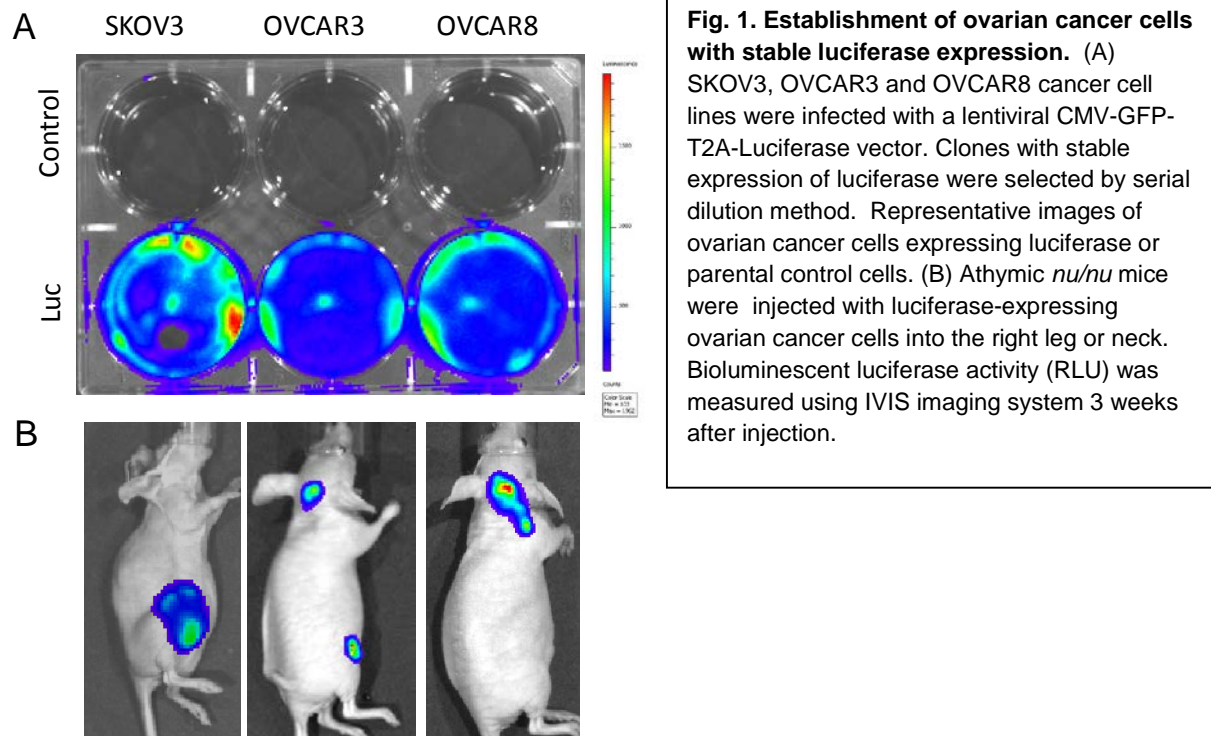
**Major Task 2:** In vivo anti-tumor study.

**Task 2-1:** Establish ovarian cancer cell lines with luciferase-expressing constructs (OVCAR3, SKOV3, & OVCAR5 cells).

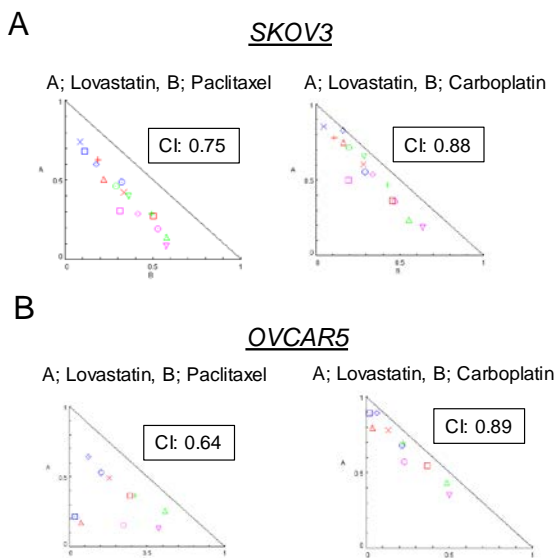
**Progress:** The proposed study is close to completion. In Fig. 1A, we demonstrated the luciferase activity measured in cultures of ovarian cancer cell lines, OVCAR3-luc, SKOV3-luc,



and OVCAR8-luc. All these three cell lines are tumorigenic in athymic *nu/nu* mice and their growth was monitored luciferase activity using a quantitative bioluminescence imaging system (IVIS Lumina imaging) (Fig. 1B).



To assess drug interactions between lovastatin and paclitaxel and between lovastatin and carboplatin, we measured combination index for both combinations in SKOV3 and OVCAR5 cell lines. Both treatment combinations had a synergistic effect, with  $CI < 1$ . Both cell lines were more sensitive to the lovastatin/carboplatin combination treatment (Fig. 2).



**Fig. 2.** Combination index plot of lovastatin and paclitaxel and lovastatin and carboplatin in SKOV3 (A) and OVCAR5 (B) cell lines. The Y-axis represents the percent of inhibition for lovastatin, and the X-axis represents the percent of inhibition for either paclitaxel or carboplatin. Combination index is less than 1 for all plots, indicating a synergistic relationship. CI is larger for lovastatin/carboplatin combination in both cell lines, indicating greater sensitivity.

Task 2-2: Purchase athymic *nu/nu* nude mice (n=120) and set ready for mouse tumor model.

Progress: Pending

Task 2-3: Perform viable surgery by orthotopic injection of ovarian cancer cells into mouse ovarian bursa.

Progress: Pending

Task 2-4: Treat the mice with lovastatin or vehicle control and monitor tumor load using live imaging system.

Progress: Pending

Task 2-5: Perform immunohistochemistry to study the expression of proliferation, autophagy, apoptosis markers, as well as other markers identified in our pilot study.

Progress: Pending

**Progress:** We plan to complete tasks 2-2 to 2-5 in the next funding year.

**Major Task 3:** Conduct gene knockdown and enzyme inhibitor study to determine which subpathway(s) is mainly responsible for the anti-tumor effects of lovastatin.

**Progress:** Tasks 3-1 to 3-4 have been accomplished and the results were recently published in *Clinical Cancer Research* (PMID: 26109099). Please also see attached paper for details.

Task 3-1: Purchased siRNAs targeting the enzymes belonging to the three subpathways in the mevalonate metabolism.

Progress: Completed

Task 3-2: Purchase small molecule enzyme inhibitors of the mevalonate subpathways.

Progress: Completed

Task 3-3: Perform gene knockdown using siRNA in ovarian cancer cell lines (same as described in Task 2). Determine the effects of lovastatin on cellular proliferation, autophagy, and apoptosis as well as the expression of new markers identified in our pilot study.

Progress: Completed

Task 3-4: Determine the effects of inhibitors on the phenotypes described in Subtask 3 in the same panel of ovarian cancer cell lines. Compare the alterations of phenotypes between 3 and subtask 4.

Progress: Completed

**Major Task 4:** Final phase: data analysis, summary, and publication

Task 4-1: Analyze and summarize data to support or refute our hypothesis. (See Task 3)

Task 4-2: Prepare manuscript(s) for journal submission. (ongoing)

**Progress:** As described above, we have recently completed Task 3 and the results were published. Our next goal is to complete the experiments proposed in Task 2 and report the findings.

**C. What opportunities for training and professional development has the project provided?**

The study provides an opportunity for a graduate student, JC Kuan, to obtain knowledge and skills in animal models of ovarian cancer and molecular and biochemical techniques for cancer biology studies. JC is a student in the epidemiology doctoral program, and this project provides him with cross-disciplinary training.

**D. How were the results disseminated to the communities of interest?**

Nothing to Report at this time.

**E. What do you plan to do during the next report period to accomplish the goals?**

**Aim1:** Complete extraction, merging and transfer of data from the multiple national datasets in Finland. Conduct any further data cleaning and categorization of covariates for our specific analysis. This is particular the case for lifetime prescription medication. We will have information for statin use, dose and frequency. Then we will conduct the proposed analysis with the goal to present data at upcoming national meetings and publish in high impact journals. This data will provide the foundation for future funding opportunities to apply to study new agents in the mevalonate pathway.

**Aim 2:** Complete the work in progress under Major Task 2 with the In vivo anti-tumor study.

**Section IV – IMPACT:**

**A. What was the impact on development of the principal disciplines(s) of the project?**

Our recently published work provides proof-of-principal evidence for an overarching question: whether persistent intake of statins can protect from gynecologic malignancies, including ovarian cancer. Our work clearly demonstrated that statin intake led to a delay in tumor progression in both genetically engineered mouse and xenograft tumor models of ovarian cancer. The result provides a biological basis for explaining the cancer protection effects of statin intake observed in epidemiology studies.

**B. What was the impact on other disciplines?**

The publication described above provides a biological basis for explaining the cancer protection effects of statin intake which we will examine further now in Aim 1, our epidemiological cohort.

**C. What was the impact on technology transfer?**

Nothing to Report at this time.

**D. What was the impact on society beyond science and technology?**

Nothing to Report at this time.

## **Section V – CHANGES/PROBLEMS:**

### **A. Changes in approach and reasons for change**

Nothing to Report at this time.

### **B. Actual or anticipated problems or delays and actions or plans to resolve**

Nothing to Report at this time.

### **C. Changes that had a significant impact on expenditures**

Nothing to Report at this time.

### **D. Significant changes in use of care of human subjects**

Nothing to Report at this time.

### **E. Significant changes in use of care of vertebrate animals**

Nothing to Report at this time.

### **F. Significant changes in use of biohazards and / or select agents**

Nothing to Report at this time.

## **Section VI – PRODUCTS:**

### **A. Publications, conferences, and presentations**

#### **1. Journal publications**

Kobayashi Y, Kashima H, Wu RC, Jung JG, Kuan JC, Gu J, Xuan J, Sokoll L, Visvanathan K, Shih IeM, Wang TL, Mevalonate Pathway Antagonist Suppresses Formation of Serous Tubal Intraepithelial Carcinoma and Ovarian Carcinoma in Mouse Models. Clinical Cancer Research. 2015 Oct15;21(20):4652-62 Epub2015 Jun24. PMID: 26109099

#### **2. Books or other non-periodical, one-time publications**

Nothing to Report at this time.

#### **3. Other publications, conference papers, and presentations**

Nothing to Report at this time.

### **B. Website(s) or other internet site(s)**

Nothing to Report at this time.

### **C. Technologies or techniques**

Nothing to Report at this time.

**D. Inventions, patent applications, and/or license**

Nothing to Report at this time.

**E. Other Products**

Nothing to Report at this time.

**Section VII – PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:****A. What individuals have worked on the project?**

Name:	Kala Visvanathan, MD
Project Role:	Principal Investigator
Research Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1.8
Contribution to Project:	Provided overall project oversight to ensure aims met according to the proposed timeline. Responsible for direct oversight conduct of Aim 1
Funding Support: (other than this award)	None
Name:	Tian-Li Wang, PhD
Project Role:	Co-Principal Investigator
Research Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1.2
Contribution to Project:	Provided oversight for the conduct of Aim 2
Funding Support: (other than this award)	None
Name:	Brenna Hogan
Project Role:	Research Data Analyst
Research Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1.0
Contribution to Project:	Assist with recoding data and data cleaning for Aim1
Funding Support: (other than this award)	None
Name:	Yusuke Kobayash, MD, PhD
Project Role:	Post Doc Research Fellow
Research Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	4.8
Contribution to Project:	Performed the experiments proposed in Aim 2
Funding Support: (other than this award)	None
Name:	Teemu Murtola, PhD, MD
Project Role:	Finnish Collaborator
Research Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	0

Contribution to Project:	Oversee data acquisition, linkage of registry data and anonymization
Funding Support: (other than this award)	At present not from this grant
Name:	Betty May, MS
Project Role:	Research Project Manager
Research Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	0.13
Contribution to Project:	Support with IRB, MTA, Data Application, Research & Confidentiality Agreement, and Invoicing
Funding Support: (other than this award)	Supported by other grants under Dr. Kala Visvanathan

**B. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report.

**C. What other organizations were involved as partners?**

Organization Name	Location	Contribution to the Project
University of Tampere	Finland	<i>Collaboration:</i> Dr. Teemu Murtola is Co-Investigator and responsible for oversight of the data acquisition, linkage of registry data and anonymization and assist Johns Hopkins with the analysis
Finnish Cancer Registry	Finland	<i>Other:</i> provide linkage to health information (ovarian cancer case data) for project
Social Insurance Institution of Finland	Finland	<i>Other:</i> provide approval for access and linkage of medical and prescriptions reimbursement data for project

**Section VIII – SPECIAL REPORTING REQUIREMENTS:**

**A. Collaborative Awards:** Not applicable

**B. Quad Charts:** Not applicable

**Section IX – APPENDICES:**

Attached is copy of publication listed above in Section VI.A.1.

# Mevalonate Pathway Antagonist Suppresses Formation of Serous Tubal Intraepithelial Carcinoma and Ovarian Carcinoma in Mouse Models

Yusuke Kobayashi<sup>1,2,3</sup>, Hiroyasu Kashima<sup>1,2</sup>, Ren-Chin Wu<sup>1,2,4</sup>, Jin-Gyoung Jung<sup>1,2</sup>, Jen-Chun Kuan<sup>1,2</sup>, Jinghua Gu<sup>5</sup>, Jianhua Xuan<sup>5</sup>, Lori Sokoll<sup>1,2</sup>, Kala Visvanathan<sup>2,6</sup>, le-Ming Shih<sup>1,2,7</sup>, and Tian-Li Wang<sup>1,2,7</sup>

## Abstract

**Purpose:** Statins are among the most frequently prescribed drugs because of their efficacy and low toxicity in treating hypercholesterolemia. Recently, statins have been reported to inhibit the proliferative activity of cancer cells, especially those with *TP53* mutations. Because *TP53* mutations occur in almost all ovarian high-grade serous carcinoma (HGSC), we determined whether statins suppressed tumor growth in animal models of ovarian cancer.

**Experimental Design:** Two ovarian cancer mouse models were used. The first one was a genetically engineered model, mogp-TAg, in which the promoter of oviduct glycoprotein-1 was used to drive the expression of SV40 T-antigen in gynecologic tissues. These mice spontaneously developed serous tubal intraepithelial carcinomas (STICs), which are known as ovarian cancer precursor lesions. The second model was a xenograft tumor model in which human ovarian cancer cells were inoculated into immunocompromised mice. Mice in both models

were treated with lovastatin, and effects on tumor growth were monitored. The molecular mechanisms underlying the antitumor effects of lovastatin were also investigated.

**Results:** Lovastatin significantly reduced the development of STICs in mogp-TAg mice and inhibited ovarian tumor growth in the mouse xenograft model. Knockdown of prenylation enzymes in the mevalonate pathway recapitulated the lovastatin-induced antiproliferative phenotype. Transcriptome analysis indicated that lovastatin affected the expression of genes associated with DNA replication, Rho/PLC signaling, glycolysis, and cholesterol biosynthesis pathways, suggesting that statins have pleiotropic effects on tumor cells.

**Conclusions:** The above results suggest that repurposing statin drugs for ovarian cancer may provide a promising strategy to prevent and manage this devastating disease. *Clin Cancer Res*; 21(20): 4652–62. ©2015 AACR.

## Introduction

The incidence and mortality of epithelial ovarian cancer in the United States has changed very little in the last 20 years; about 22,000 women will receive a new diagnosis this year. Because of the aggressiveness of the disease, once diagnosed, the overall

5-year survival rate is expected to be less than 50%. Part of the problem is that it is difficult to detect ovarian cancer at early stages, and when diagnosed at late stages, there are few effective treatments. Hence, it is critical to develop preventive strategies to reduce the risk of this disease. Currently, for women who are *BRCA* mutation carriers, bilateral salpingo-oophorectomy (BSO) is the recommended surgical procedure to protect these women from developing ovarian cancer. In addition, oral contraceptives, which reduce the frequency of ovulation, have been shown to be effective in reducing the incidence and mortality of ovarian cancer (1). However, neither of these approaches is without concern. Oral contraceptive use is not as safe in older women because the risk of thrombosis increases with age (2); furthermore, its use increases the risk of breast cancer and cervical cancer (3). On the other hand, patients who undergo bilateral salpingo-oophorectomy may suffer from postsurgery complications, especially those symptoms associated with decreased estrogen levels, which include increased adiposity, cardiovascular disease, osteoporosis, and depression at relatively young ages (4–7). Therefore, safer and more cost-effective chemopreventive strategies aimed at preventing or delaying the development of epithelial ovarian cancer are urgently needed.

One potential approach toward chemoprevention of ovarian cancer is to repurpose existing drugs that have been frequently

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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**doi:** 10.1158/1078-0432.CCR-14-3368

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## Translational Relevance

Recent studies have led to a paradigm shift in our conceptualization of the cellular origin of ovarian high-grade serous carcinomas (HGSC), the most common and aggressive type of ovarian cancer. It appears that many HGSCs, traditionally classified as ovarian in origin, actually originate from the distal fallopian tube where precursor lesions, serous tubal intraepithelial carcinoma (STIC), can be identified. We used a genetically engineered mouse model that faithfully recapitulates STIC and ovarian tumor progression to determine whether statin intake can prevent the development of STIC. We provide new evidence that lovastatin treatment suppresses STIC development in this mouse model. Furthermore, when applying lovastatin treatment to a xenograft model of ovarian cancer, it efficiently reduces tumor progression. We also elucidate the manifold mechanisms by which statins exert the observed antitumor effects. As statins have been widely prescribed to prevent cardiovascular disease and exhibit low toxicity in patients, our results warrant further investigation to determine the clinical benefit of statins in preventing and treating ovarian cancer.

prescribed to treat non-cancer-related medical conditions in a large population. We can take advantage of the existing population-based database to determine their potency in cancer control. One such class of drugs is statins, which target 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway, which are widely used to prevent and treat hypercholesterolemia. The reasons to focus on statins are multifold. First, it has been recently reported that statins can inhibit the proliferative activity of cancer cells, especially those with *TP53* mutations (8). *TP53* mutations occur in virtually all ovarian high-grade serous carcinoma (HGSC) and in its precursor lesion, serous tubal intraepithelial carcinoma (STIC), suggesting that statins may be effective on HGSC and STIC. Second, statins have been in clinical use for a significant period of time and are well tolerated in patients. Known side effects including alterations in liver function and muscle weakness or tenderness occur only in a small fraction of patients (9). Third, most statins are off-patent generic drugs, offering an inexpensive option as anticancer agents. Fourth, higher circulating levels of low-density lipoprotein (LDL), which can be treated with statins, have been associated with reduced survival among ovarian cancer patients compared with those with LDL levels in the normal range (10). Fifth, statin use is about 11% in the overall U.S. population and as high as 44% in people above 65 years (11). Therefore, there should be collections of existing population-based data to permit researchers to investigate cancer risk or mortality among statin users.

That being said, the data from individual observational studies have been inconsistent and are limited with respect to sample size and lack of detailed information about statin use. The largest study to date included 4,103 epithelial ovarian cancer patients from Danish nationwide registries and observed no difference in ovarian cancer incidence among statin users (12). However, there were only 320 women with HGSC enrolled in this study who were on statins. Contrarily, a recent meta-analysis of 14 studies that included cohort, case-control, and randomized controlled trials,

statin use was associated with a 21% reduction in ovarian cancer risk, and there was no significant heterogeneity among studies (13). To date, only two observational studies (with 150 cases or fewer) have been published that examined the association between statin use and ovarian cancer mortality (14, 15). Both reported a 50% reduction in ovarian cancer mortality among statin users. In a prospective study that examined statin use and mortality from all cancers, a reduction was also observed among women with epithelial ovarian cancer, but the point estimate was not statistically significant (16). Given the discordant results in these reports, it appears important to demonstrate the biologic effects of statins in well-controlled studies such as in animal models of ovarian cancer.

Herein, we determined the antitumor effects of lovastatin, a lipophilic statin, in two animal models of ovarian cancer. The first model is a genetically engineered mouse model, *mogp*-TAG, in which the promoter of oviduct glycoprotein 1 (*OVGP1*) is used to drive expression of the SV40 T antigen in gynecologic tissues (17). These mice spontaneously develop STICs and ovarian/tubal carcinomas at a relatively young age. This transgenic mouse model displays a stepwise progression from normal tubal epithelium to invasive epithelial ovarian cancer, simulating the pathogenesis in humans (18). The second model is a xenograft tumor model in which human ovarian cancer cells are inoculated into immunodeficiency mice. Using the transgenic mouse model, we determined the capacity of lovastatin, as a chemopreventive agent, to suppress the formation of STICs. Using the xenograft model, we assessed the potency of lovastatin in delaying the growth of ovarian tumors. We also explored the molecular mechanisms underlying the antitumor effects of lovastatin.

## Materials and Methods

### Animal studies

The generation of the *mogp*-TAG transgenic mouse has been described previously (17). Mice were housed and handled according to a protocol approved by the Johns Hopkins University (JHU, Baltimore, MD) Animal Care and Use Committee. The genotype of the *mogp*-TAG transgene was confirmed by tail DNA extraction and polymerase chain reaction (PCR). PCR was performed using the following conditions: denaturation at 94°C for 30 seconds, followed by 30 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 45 seconds, and a final extension at 68°C for 5 minutes. The primer sequences were: forward—GAAATGGAAGATG-GAGTAAA–; reverse—AATAGCAAAGCAAGCAAGAGT–. *mogp*-TAG mice were treated daily with 50 or 100 mg/kg lovastatin diluted in 0.5% methylcellulose by gastric intubation using disposable feeding tubes beginning at 3 weeks of age and continued until euthanasia at 8 weeks. Reproductive tracts were removed, weighed, formalin-fixed, and embedded in paraffin. Because tumor cells occupy approximately 75% of the total mass of the female genital tract in untreated mice, tissue weight was used as an indicator of tumor burden.

To test the therapeutic potential of lovastatin in xenograft tumor models, human ovarian cancer cells, SKOV3-IP or OVCAR5 cells ( $5 \times 10^6$ ), were injected subcutaneously into the left flank of 6-week-old female mice. The mice were randomly assigned to treatment or control groups; beginning 1 week after tumor cell inoculation, lovastatin (12.5 mg/kg per injection) was administered via intraperitoneal (i.p.) injection twice weekly; atorvastatin (10 mg/kg per injection) was i.p. administered daily (19). Tumor



diameters were measured twice per week using a caliper. Tumor volume ( $V$ ) was calculated using the formula:  $V = A \times B^2/2$  (where  $A$  = axial diameter;  $B$  = rotational diameter). Excised tumors were homogenized for RNA extraction or were fixed overnight in neutral-buffered formalin and embedded in paraffin blocks.

#### Cell culture and siRNA transfection

The cell lines used in this study, including SKOV3 and OVCAR5, were purchased from the American Type Culture Collection (ATCC). SKOV3-IP is a derivative line of SKOV3 after three passages in athymic nude mice and is potentially tumorigenic. All cell lines were cultured at 37°C, 5% CO<sub>2</sub> in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 U/mL). Cell line authentication was verified by the STR test performed at the Genetic Resources Core Facility at JHU. The STR profiles of SKOV3 and OVCAR5 matched 100% with the published references. The STR profile of SKOV3-IP exhibited 97% match with the SKOV3 profile provided by the ATCC. Lovastatin was used in the *in vitro* experiments. A pilot metabolomics study performed on lovastatin-treated OVCAR3 cell cultures demonstrated that lovastatin potentially suppressed the activity of HMG-CoA reductase, resulting in the accumulation of HMG-CoA metabolites in cultured cells.

For gene silencing studies, gene-specific Stealth siRNAs and medium GC control siRNA were purchased from Invitrogen. RNAi duplexes were transfected into ovarian cancer cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Final siRNA concentrations were 50 nmol/L. Viable cells were counted using a T20 automatic cell counter (Bio-Rad).

#### Western blot analysis

Tumor tissues or cells were homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% NP40) with Halt Protease Inhibitor Cocktail (1861278, Thermo Fisher Scientific). Protein concentration in tissues or cell lysates was determined with a protein assay kit (Bio-Rad) using bovine serum albumin as a standard. Aliquots of protein lysate (30 µg) were separated by SDS-PAGE, and Western blot analyses were performed using standard procedures. Blots were developed using the Amersham ECL Western Blotting Detection Reagents kit (GE Healthcare UK Ltd.). Primary antibodies used in this study include LC3A (#4599; Cell Signaling Technology, Inc.), LC3B (#3868; Cell Signaling Technology), cleaved caspase-3 (#9664; Cell Signaling Technology), PARP (#5625; Cell Signaling Technology), PCNA (sc-56; Santa Cruz Biotechnology), and GAPDH (#5174; Cell Signaling Technology).

#### Immunohistochemistry

Paraffin-embedded tissue sections (4 µm) were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was performed by incubating tissue sections with Trilogy (Cell Marque; catalog no. CMX833). Endogenous peroxidase activity was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub> for 15 minutes. Sections were preincubated with Dako Antibody Diluent (Dako) at room temperature for 30 minutes, followed by incubation with antibody diluted in Dako Antibody diluent at 4°C overnight. Positive reactions were detected by applying EnVision+/HRP polymer (Dako) for 30 minutes, followed by incubation in DAB substrate for 5 minutes (Liquid DAB+; Dako). The slides were then counterstained with hematoxylin to visualize the cell nuclei. Antibodies used were: human Ki-67 (catalog no. M7240; Dako), mouse Ki-67

(catalog no. 12202; Cell Signaling Technology), LAMC1 (catalog no. HPA001908; Sigma-Aldrich), and phospho-Histone H3 (Ser10) antibody (catalog no. 9701; Cell Signaling Technology).

#### Quantification of immunohistochemical staining

Ki-67 or LAMC1 positivity in fallopian tubal epithelium of mogp-TAg mice was quantitated as the percentage of positively stained cells. At least 3,200 tubal epithelial cells were counted in each sample.

The proliferative index in xenografts was quantitated as the percentage of Ki-67 positively stained epithelial cells. The total number of epithelial cells and the number of positively stained epithelial cells were counted in each microscopic field (magnification, ×40; Nikon Orthoplan microscope). At least 10 random fields (greater than 2,500 tubal epithelial cells) per experimental group were scored by two independent observers who were blinded to the treatment group. Differences in counts between the observers were <10%.

#### Microarray analysis

Quality and quantity of total RNA was determined using an Agilent 2100 Bioanalyzer and a NanoDrop spectrophotometer, respectively. cRNA was synthesized using an Illumina RNA amplification kit (Ambion) following the procedure suggested by the manufacturer. BeadChip hybridization was performed according to the manufacturer's instruction. Arrays were scanned on an Illumina BeadStation 500. BeadChip array data quality control was performed using Illumina BeadStudio software. Probe average intensity signal was calculated with BeadStudio without background correction. Empirical Bayes method (R package limma) was applied to assess the differential expression between DMSO- and statin-treated cells. Differentially expressed probes were defined as having fold change greater than 1.7 and adjusted  $P < 0.05$  (false discovery rate).

Microarray data were deposited in the GEO repositories (accession number GSE68986).

#### Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed on gene expression microarray data using GSEA desktop application v2.0.14 (<http://www.broad.mit.edu/gsea/>) and KEGG gene sets from the Molecular Signature Database (MSigDB) version 4.0 (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>). After ranking genes according to log<sub>2</sub> ratio of expression (control/statin), enrichment scores and significance were calculated by GSEA using 2,000 permutations of each gene set.

#### Quantitative RT-PCR analysis

RNA was isolated using the RNeasy kit from Qiagen. Total cellular RNA was reverse transcribed into cDNA using an iScript cDNA kit (Bio-Rad). Real-time reverse transcription-PCR (RT-PCR) was performed on a CFX96 iCycler (Bio-Rad) using the SYBR Green I detection method (Invitrogen). Primer sequences are listed in Supplementary Table S1. Relative quantitation of mRNA levels was plotted as fold increases compared with untreated samples. Actin B expression was used for normalization.  $\Delta C_t$  value (target gene  $C_t$  minus Actin B  $C_t$ ) was averaged from three replicate wells per sample, and  $\Delta\Delta C_t$  was calculated as the difference between statin treatment and vehicle control in the same cell line. Relative mRNA quantity was calculated using the  $2^{-\Delta\Delta C_t}$  formula.

### Analysis of plasma cholesterol and triglycerides

Mice were euthanized at the end of study. Blood was collected by intracardiac aspiration using a 1-mL syringe with a 25-gauge needle and placed in a microcentrifuge tube containing EDTA. Blood was centrifuged, plasma isolated, and cholesterol and triglycerides measured using standard clinical laboratory assays on a Roche Hitachi Cobas c701 analyzer (Roche Diagnostics).

### Statistical analysis

Statistical analyses were performed with Prism 5.0 GraphPad software. The Mann–Whitney *U* test was performed to assess tumor volume and immunohistochemical data for LAMC-1, Ki-67, and phospho-Histone H3 for vehicle- and lovastatin-treated groups. Specific analyses performed for each assessment are described in the results and figure legends. In all analyses, data were evaluated using a two-tailed test;  $P < 0.05$  was considered statistically significant.

## Results

### Lovastatin suppresses formation of STICs, precursor lesions of ovarian cancer

To determine whether pharmacologic inhibition of the mevalonate pathway prevents tumor development, we used a genetically engineered mouse model that expresses the SV40 large T antigen driven by the oviduct glycoprotein 1 (*OVGP1*) promoter (mogp-Tag transgenic mice; ref. 17). Mogp-Tag mice consistently develop spontaneous STIC, the precursor lesion of most HGSCs, and uterine stromal sarcoma at 6–7 weeks of age (18). The mice were treated with lovastatin (50 or 100 mg/kg) or control vehicle beginning at 3 weeks of age. The animals were euthanized at 8 weeks to evaluate tumor burden. We found that treatment with lovastatin at either dose (50 or 100 mg/kg) significantly reduced the total tumor mass in the female reproductive tract, as evidenced by the organ weight (Supplementary Fig. S1). Plasma levels of cholesterol and triglycerides were measured at the endpoint, and the data showed significantly reduced cholesterol levels and marginally reduced triglyceride levels in mice treated with lovastatin as compared with controls (Supplementary Fig. S2A).

To determine whether lovastatin reduced the formation and extent of STICs, we compared the histopathology of the fallopian tubes between lovastatin-treated and vehicle-treated mice. Histopathology of fallopian tube section from a representative lovastatin-treated mouse exhibited normal-appearing morphology (top left, Fig. 1A). In contrast, fallopian tube sections from control vehicle-treated mice contained either morphologic features of STICs (top right and bottom left, Fig. 1A) or a tubal carcinoma (bottom right, Fig. 1A). Mice treated with lovastatin exhibited fewer and smaller foci of STICs in individual fallopian tubes than did vehicle-treated mice. At high magnification, STIC from a control mouse could be seen to contain pseudo-stratified epithelial cells with enlarged and atypical nuclei as well as mitoses (right, Fig. 1B), features that were absent in a normal-appearing tube section from a statin-treated mouse (left, Fig. 1B). We also quantified STICs in tissue sections by immunohistochemistry using a STIC-associated marker, laminin C1 (20). We found that the percentage of laminin C1-positive tubal epithelial cells in lovastatin-treated mice was significantly reduced compared with that of the vehicle control group (Fig. 1C and D). Similarly, based on Ki-67 indices, the proliferative activity in STICs of the lova-

statin-treated mice was significantly decreased compared with that of the control group (Fig. 1C and D).

The two different doses of lovastatin used in this study appeared to be safe in animals. The body weights of mice were similar between the two groups (Supplementary Fig. S2A). There was no evidence of lethargy or other physical compromise. Necropsy was performed at the endpoint, and histopathologic examination of internal organs including liver, spleen, kidney, heart, intestine, and brain did not reveal tissue damage in lovastatin-treated mice.

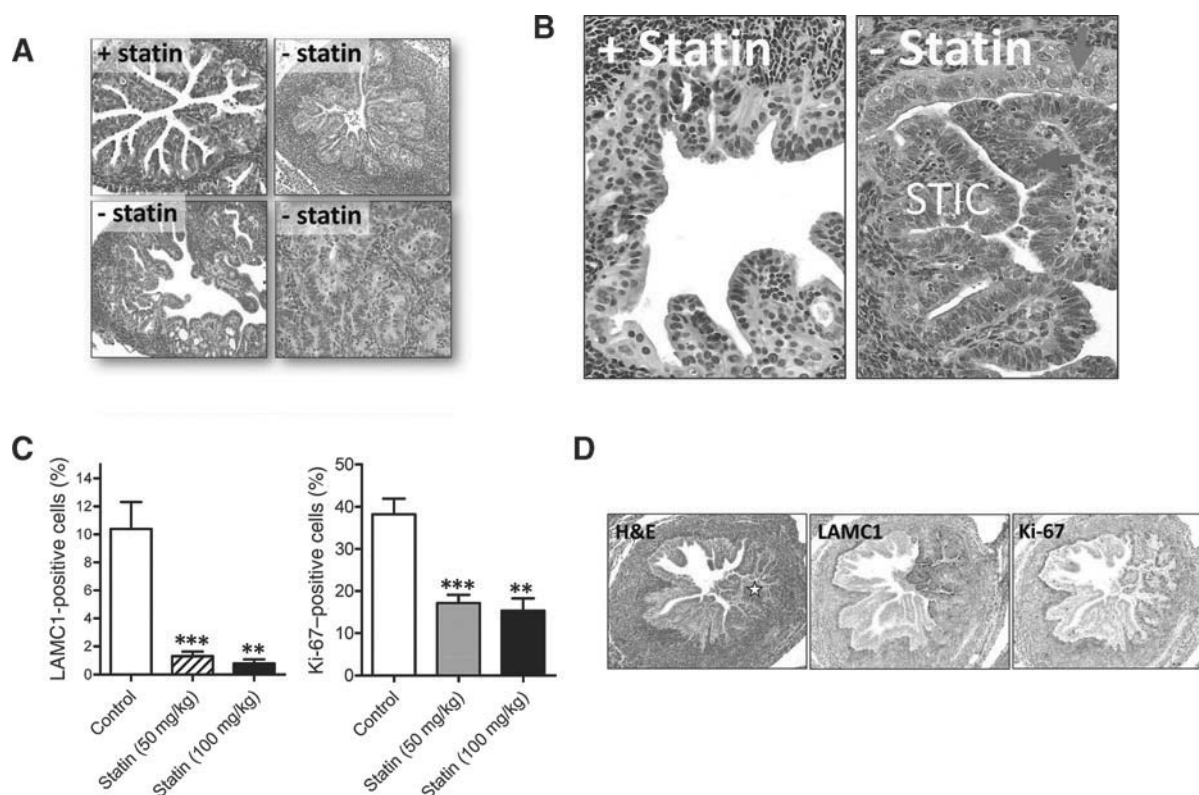
### Lovastatin inhibits tumor growth in xenograft models of human ovarian cancer

The studies in the genetically engineered mouse model demonstrated the potency of lovastatin in suppressing spontaneously developing STICs. Next, we determined whether lovastatin exerted antitumor effects on xenograft mouse models of ovarian cancer. SKOV3-IP or OVCAR5 ovarian cancer cells were inoculated subcutaneously into athymic nude mice. Beginning 1 week after tumor inoculation, lovastatin (12.5 mg/kg) or vehicle control was administered by intraperitoneal injection twice a week for 4 weeks. All mice were evaluated for tumor growth twice a week until day 28, when animals were euthanized for endpoint study of tumor burden. The lovastatin treatment was well tolerated by the mice, and there was no effect on body weight measured at the endpoint (Supplementary Fig. S2B and S2C). Blood cholesterol and triglycerides levels were significantly reduced by lovastatin treatment (Mann–Whitney *U* test; Supplementary Fig. S2B and S2C). Furthermore, lovastatin administration significantly reduced the rate of tumor growth in both SKOV3-IP and OVCAR5 tumor xenografts (Fig. 2, left). Immunohistochemistry was performed on the excised tumors using antibodies to Ki-67 (a proliferation marker) and to Ser-11 phosphorylated histone 3 (a mitosis marker). The data showed that tumors from lovastatin-treated animals had significantly fewer proliferating cells than did tumors from vehicle-treated animals (Mann–Whitney *U* test; Fig. 2, middle). The number of mitotic cells was significantly reduced in the lovastatin-treated mice in the OVCAR5 model, while it was marginally reduced in the SKOV3-IP model (Mann–Whitney *U* test; Fig. 2, right).

To determine whether other lipophilic statins exerted an anti-tumor phenotype, we assessed another inhibitor of HMG-CoA reductase, atorvastatin (Brand name: Lipitor), in an OVCAR5 tumor xenograft model. Daily injections of atorvastatin (10 mg/kg) led to significantly reduced tumor sizes as compared with vehicle control treatment (Supplementary Fig. S3A;  $P < 0.01$ , Mann–Whitney *U* test). Similar to lovastatin, atorvastatin treatment led to reduction in proliferative and mitotic activities as assessed by PCNA and phospho-histone H3 expression levels, respectively, in tumor tissues as compared with vehicle control treatment (Supplementary Fig. S3B).

### Effects of lovastatin on autophagy, cellular proliferation, and apoptosis in ovarian cancer cells

We next tested whether statin treatment affected autophagy and apoptosis after lovastatin treatment in SKOV3 and OVCAR5 cell cultures. Cells were incubated with 10  $\mu\text{mol/L}$  lovastatin or vehicle control for 0, 6, 12, 24, 36, or 48 hours, and the expression levels of markers of autophagy and apoptosis were determined by Western blot analysis. LC3A and LC3B are two isoforms



**Figure 1.**

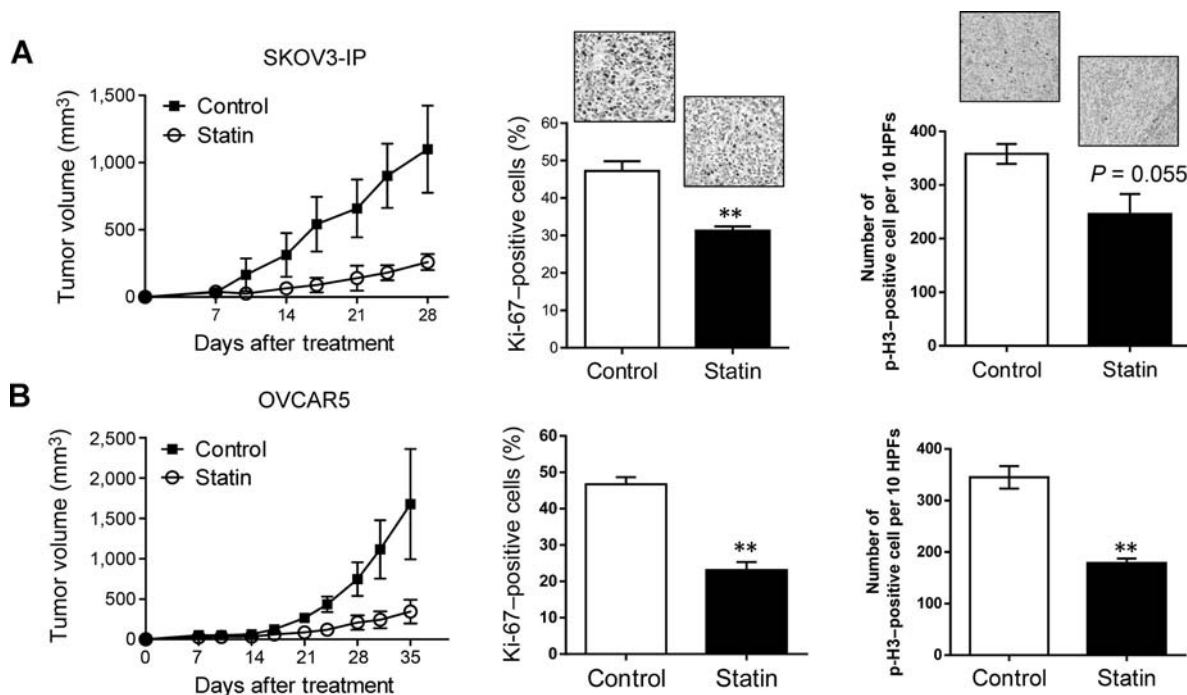
Lovastatin suppresses the formation of STICs, the precursors of many ovarian HGSCs. A, representative photomicrographs of fallopian tube section from a statin-treated mogp-TAg transgenic mouse showing normal-appearing morphology, whereas extensive STICs are observed in fallopian tube sections from the vehicle-treated mogp-TAg mice. In one of the control mice (bottom right), a tubal carcinoma is also noted. B, higher magnification of fallopian tube sections from statin-treated versus vehicle-treated mice. Red arrow, normal-appearing fallopian tube epithelium; blue arrow, STIC. C, summary of LAMC-1 and Ki-67 staining results. Bar graphs depict the percentage of LAMC-1-positive or Ki-67-positive epithelial cells among total fallopian tube epithelial cells per section. In each experiential group, data were collected from 10 representative sections from each mouse; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ , two-tailed Mann-Whitney  $U$  test. D, representative images of H&E, LAMC-1, and Ki-67 staining on tissue sections from fallopian tubes of mogp-TAg mice. Star indicates the STICs.

of microtubule-associated protein 1 light chain, LC3, which undergoes posttranslational modification during autophagy. Cleavage of LC3 at the C-terminus yields cytosolic LC3-I. During autophagy, LC3-I is converted to LC3-II through lipidation, which allows LC3 to become associated with autophagosomes. The conversion of LC3-I to faster-migrating LC3-II was used as an indicator of autophagy. Activity of autophagy based on LC3A-II and LC3B-II expression was detected as early as 12 hours after statin exposure, while apoptosis as demonstrated by cleavage of caspase-3 and PARP1 was undetected until 36 hours after statin treatment (Fig. 3A). Next, we performed cell-cycle analysis in lovastatin-treated SKOV3 and OVCAR5 cells using flow cytometry. Lovastatin treatment resulted in a significant, dose-dependent accumulation of ovarian cancer cells in  $G_0$ – $G_1$  phase, which was accompanied by a concomitant decrease in the number of cells in  $G_2$ –M phase (Fig. 3B). We next attempted to assess autophagy and apoptosis in the tumor xenografts and found that autophagy markers, LC3A-II and LC3B-II, were more abundant in OVCAR5 and SKOV3-IP tumor xenografts in the statin-treated group than in the vehicle-treated group (Fig. 3C). Because increased levels of LC3-I were also observed in statin-treated tumor xenografts, qRT-PCR was performed in these tumors to determine whether LC3 transcript levels were altered by statin treatment. The results showed that both LC3A and LC3B mRNA levels were elevated

in tumors derived from statin-treated mice as compared with tumors excised from control vehicle-treated mice (Mann-Whitney  $U$  test; Supplementary Fig. S4). This finding suggests that there is an increased demand of autophagy under statin-induced conditions. As a result, not only was the lipidized LC3 increased, but its transcript and protein levels were also elevated. In contrast, apoptosis appeared to be an inconsistent event in the tumor xenografts because the expression levels of cleaved caspase-3 and cleaved PARP1 varied among different xenografts (data not shown).

#### Lovastatin affects expression of genes involved in DNA replication, Ras/Rho signaling, and cholesterol biosynthesis

To elucidate the molecular mechanisms leading to the observed antitumor effects of lovastatin, we performed global gene expression analysis using the Illumina Bead Array in ovarian cancer cell cultures that had been treated with 10  $\mu$ mol/L lovastatin or control vehicle for 48 hours. We observed differential expression of 1,309 genes in OVCAR5 and 4,128 genes in SKOV3 following lovastatin treatment (fold change  $> 1.7$  and FDR  $< 0.05$ ). Of these, 693 genes overlapped between OVCAR5 and SKOV3 ( $P = 1.5 \times 10^{-268}$ ; hypergeometric test). Ingenuity Pathway Analysis (Qiagen) demonstrated that the most frequently involved canonical pathways included cell-cycle control of DNA



**Figure 2.**

Lovastatin suppresses growth of human ovarian tumor xenografts. SKOV3-IP (A) or OVCAR5 (B) cells were injected subcutaneously into athymic nude mice. One week after tumor inoculation, mice received i.p. treatment with lovastatin (12.5 mg/kg) twice a week until termination of the study. Tumor volume was measured by a caliper twice per week. The mean tumor volumes are plotted  $\pm$  SD ( $n = 5$  for each group). Middle, bar graphs depict the percentage of Ki-67-positive cells per high-power field ( $\times 400$ ). Five high-power ( $\times 400$ ) fields were screened per tumor. In total, 25 high-power fields were included for each experimental group of 5 mice. \*\*,  $P < 0.01$ , two-tailed Mann-Whitney  $U$  test. Right, bar graphs depict the average number of phospho-Histone H3-positive cells per high-power field ( $\times 400$ ). Ten high-power ( $\times 400$ ) fields were collected from each tumor, and in total, 50 high-power fields were included for each experimental group. \*\*,  $P < 0.01$ , two-tailed Mann-Whitney  $U$  test. For SKOV3-IP,  $P = 0.055$ .

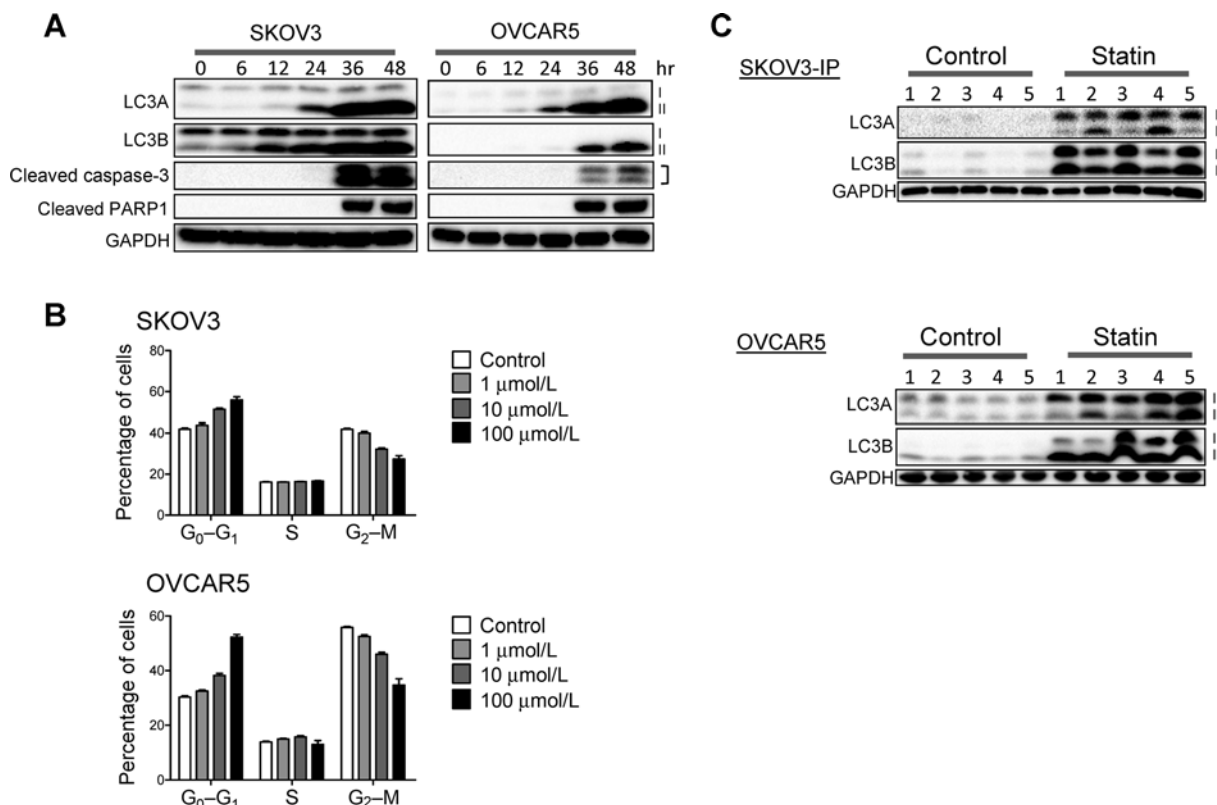
replication and phospholipase C (PLC) signaling (Fig. 4A and Table 1). In addition, several members of the Rho and Ras small G protein families are within the PLC signaling pathway. We also performed GSEA to determine the enrichment of KEGG functional pathways in our microarray data. The GSEA results were in agreement with the IPA pathway analysis; again, the gene set involving DNA replication ranked at the top of the list (Supplementary Table S2; Fig. 4B). Interestingly, we observed that several genes in the mevalonate pathway including HMGCS1 and HMGCR were upregulated in cells treated with lovastatin, suggesting that tumor cells responded to mevalonate pathway blockage by transcriptionally upregulating genes in the same pathway to compensate for the reduced pools of pathway metabolites. Similar regulation of enzymatic activity by transcription in response to metabolite levels in the same pathway is well documented, and is conserved from yeast to mammals (21). Furthermore, a significant fraction of genes in the glycolysis/gluconeogenesis pathway was upregulated in cells treated with lovastatin (Fig. 4C and Supplementary Table S3). Four of the genes including PC, ENO2, ENO3, and HKDC1 in the glycolysis/gluconeogenesis pathway were upregulated in both cell lines following lovastatin treatment (Fig. 4C).

To confirm expression changes induced by lovastatin treatment, we used qRT-PCR to assess mRNA expression of several members in the DNA replication and mevalonate biosynthesis pathways in tumor xenografts as well as in tumors derived from mogp-TAg mice (Fig. 4D). Expression levels of MCM2-7 and

MCM10, which encode minichromosome maintenance (MCM) proteins essential for initiation and elongation of DNA replication, were consistently downregulated in lovastatin-treated tumors (Fig. 4D). In contrast, expression of HMGCS1 and HMGCR, which, as indicated above, encode enzymes in the mevalonate pathway, were significantly upregulated in lovastatin-treated tumors as compared with tumors from control-treated mice (Fig. 4D).

#### Protein prenylation mediates the antiproliferative phenotype of lovastatin

To determine whether metabolites in the mevalonate pathway (see Supplementary Fig. S5 for pathway outline), including cholesterol, coenzyme Q10 (CoQ10), geranylgeranyl pyrophosphate (GGPP), or farnesyl pyrophosphate (FPP), could rescue lovastatin-induced antiproliferative effects in ovarian cancer cells, we cotreated OVCAR5 and SKOV3 cells with lovastatin (10  $\mu$ M/L) and individual metabolites. The addition of GGPP significantly reverted the antiproliferative effect of lovastatin (Fig. 5A and B), while applying GGPP or FPP as a single agent did not affect proliferation (Supplementary Fig. S6). In contrast, coinubation of ovarian cancer cells with lovastatin and FPP, water-soluble cholesterol, or CoQ10 had no effect on lovastatin-induced antiproliferative effects (Fig. 5A and B). These data suggest that the antiproliferative effect of lovastatin is likely mediated by depletion of endogenous GGPP pools and is less likely to be related to cholesterol.

**Figure 3.**

Lovastatin induces autophagy and cell-cycle arrest in ovarian cancer cells. A, SKOV3 and OVCAR5 cell cultures were incubated with lovastatin or vehicle control for various times and were harvested for Western blot analysis to detect autophagy (LC3A and LC3B) and apoptosis (cleaved caspase-3 and PARP-1). I: LC3-I; II: LC3-II; bracket: cleaved caspase-3. B, SKOV3 and OVCAR5 cells were treated with 0, 1, 10, or 100 μmol/L lovastatin for 48 hours. Cell cycle was measured by flow cytometry using propidium iodide (PI) staining. The percentages of cells in G<sub>0</sub>-G<sub>1</sub>, S, and G<sub>2</sub>-M phases are depicted. C, SKOV3-IP and OVCAR5 xenograft tumors from control and lovastatin-treated mice were excised, lysed, and analyzed by Western blotting using antibodies against LC3A and LC3B to detect autophagy. Blots were stripped and reprobed with GAPDH antibody to verify equal protein loading. Each lane represents a different xenograft tumor sample.

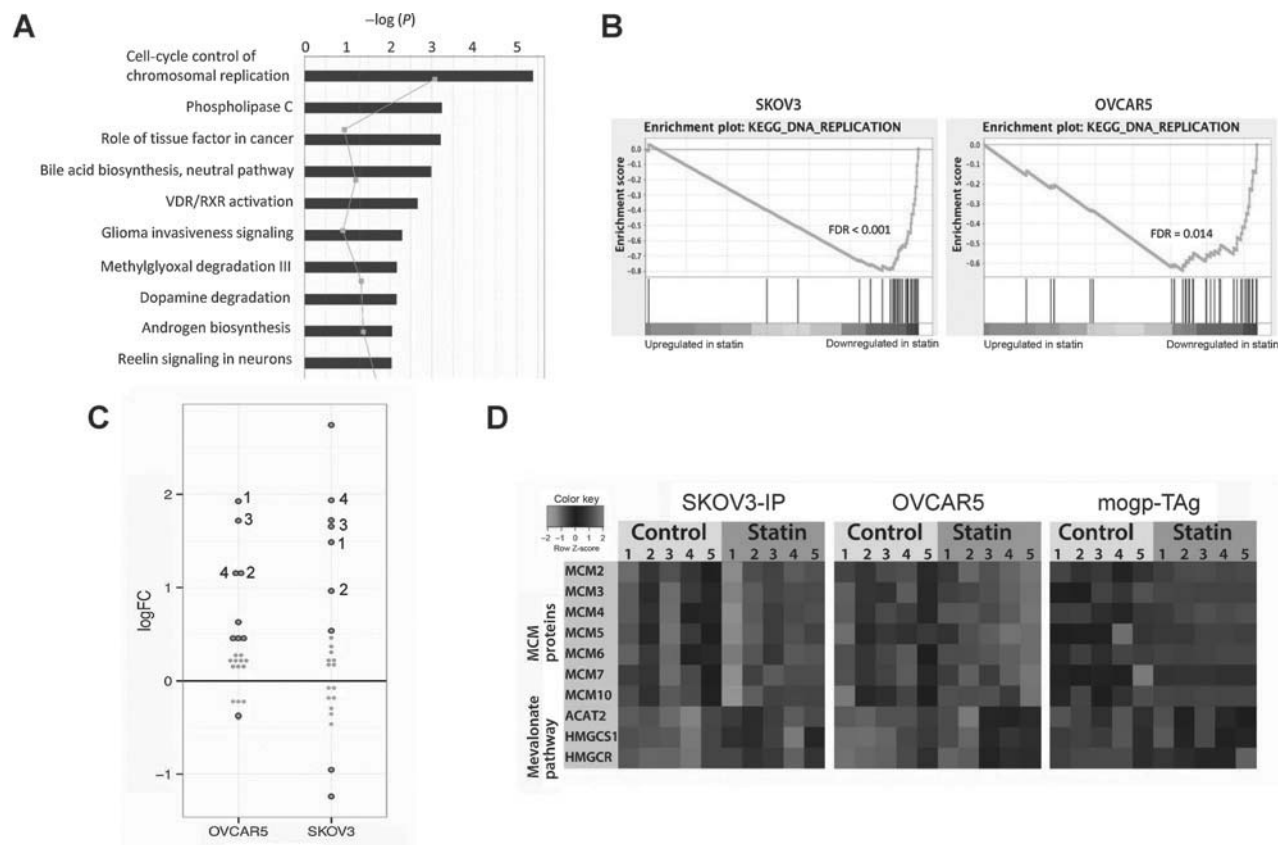
Because the above rescue assay indicated that the geranylgeranylation subpathway was involved in the cytotoxic effect of statin, we used an RNAi approach to further dissect key enzymes in this subpathway (see Supplementary Fig. S5 for pathway scheme). The expression of geranylgeranyltransferases, including PGGT1B and RABGGTB, was downregulated in SKOV3 and OVCAR5 cells by two different siRNAs targeting each enzyme. As a negative control, cells were transfected with nontargeting siRNAs. The knockdown efficiency of each target gene was confirmed by qRT-PCR (Supplementary Fig. S7). Squalene synthase (FDFT1), a critical enzyme in the cholesterol synthesis subpathway, was also included as an experimental control. Knockdown of PGGT1B or RABGGTB significantly reduced proliferation, while knockdown of FDFT1 did not have a detectable effect (Fig. 5C and D).

## Discussion

Although anticancer actions of statins in both ovarian tumor cell culture and xenograft models have been reported previously (22–24), the chemopreventive potential of statins in spontaneous ovarian/fallopian tube mouse tumor models has not been previously assessed. We report that lovastatin treatment prevents the formation of ovarian cancer precursors—STICs—in mogp-TAg

transgenic mice. In addition, we demonstrate that lovastatin also reduces the tumor volume of ovarian tumor xenografts. As statin drugs have been widely prescribed to prevent cardiovascular disease and exhibit low toxicity in patients, our results warrant further investigation to determine the clinical benefit of statins in preventing ovarian cancer and in treating advanced-stage ovarian cancer.

The antitumor effect of statins is likely mediated by multiple mechanisms. Statins have been reported to modulate local inflammatory responses; when applied to the tumor microenvironment, this mechanism may help control tumor growth (25). In support of this view, bisphosphonate, a mevalonate pathway blocker, has been recently reported to be uptaken by the tumor-associated macrophages in breast cancer tissues with calcification and the drug may specifically target this type of immune cells (26). On the other hand, previous studies have shown that protein modification by geranylgeranylation is critical for the antiproliferative and/or apoptotic activity of statins on tumor cells (8, 27–30). Geranylgeranylation involves the covalent addition of the GGPP lipid to a conserved motif on proteins and is an essential step in controlling membrane localization. For members of the Rho/Rab or phospholipase superfamilies, geranylgeranylation specifies their localization to cellular membranes, a critical step for signaling activation (31). Rho GTPases are closely involved in



**Figure 4.**

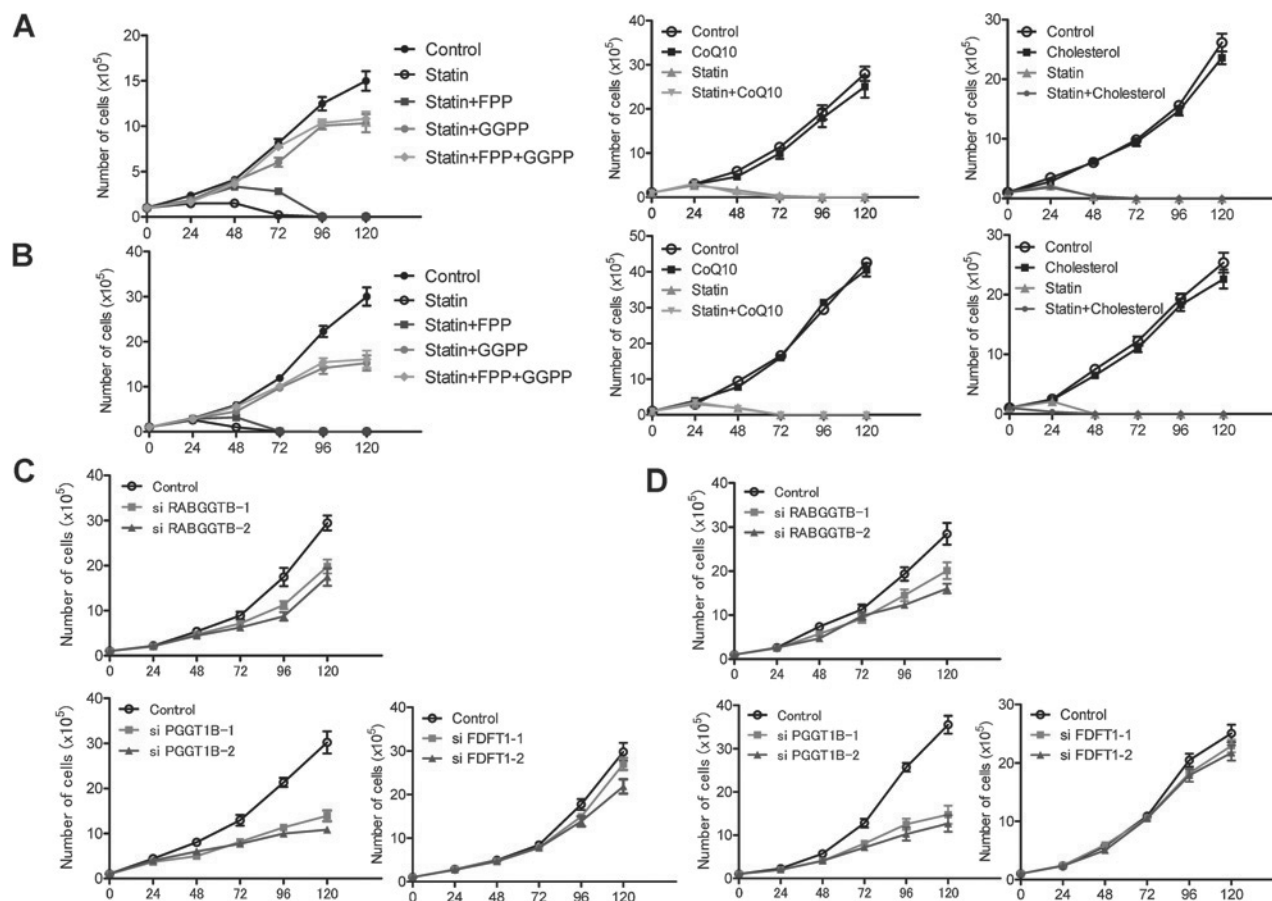
Genome-wide expression profiling of lovastatin-regulated genes using *in vitro* and *in vivo* tumor models. A, canonical pathways of statin-regulated genes revealed by Ingenuity Pathway Analysis. B, lovastatin-regulated genes in SKOV3 and OVCAR5 tumor cells were compared with the KEGG functional pathways to evaluate the enriched gene sets. Shown is the top downregulated gene set, DNA replication. C, expression levels of genes in the glycolysis/gluconeogenesis pathway. Red circle, genes upregulated by lovastatin; blue circle, genes downregulated by lovastatin; circles with black outlines indicate that the differential expressions are statistically significant. Numbers represent genes that are upregulated in both cell lines; 1, 2, 3, and 4 indicate ENO2, ENO3, HKDC1, and PC, respectively. The relative expression values of each data circle can be found in Supplementary Table S3. D, qRT-PCR analysis of expression of genes in DNA replication and sterol biosynthesis pathways in SKOV3-IP and OVCAR5 tumor xenografts and in spontaneous tumors derived from mogp-TAG mice. Normalized expression values derived from three replicates from each sample are shown; green pseudocolor coding represents downregulation, and red coding represents upregulation.

cancer cell morphogenesis, motility, and migration. Rab GTPases control membrane and vesicle trafficking. Phospholipases (PLC, PLD, and PLA) are essential mediators of intracellular signaling and regulate multiple cellular processes that can promote tumorigenesis (32). Given the functional role of geranylgeranylation in

regulating these important signaling pathways in human cancer, inhibition of protein geranylgeranylation is considered a promising target for cancer treatment. Our knockdown study showing that enzymes involved in geranylgeranylation are critical for tumor cell growth further strengthens this view. In addition,

**Table 1.** IPA pathways regulated by lovastatin in ovarian cancer cells

Ingenuity canonical pathways	$-\log(P)$	Ratio	Molecules
Cell cycle control of chromosomal replication	5.38E+00	2.35E-01	MCM5, MCM3, MCM6, MCM2, CDT1, CDC6, ORC6, MCM4
PLC signaling	3.23E+00	7.17E-02	ARHGEF4, PLD3, RRAS, PLA2G4C, MEF2A, ITGA5, CREB5, HMOX1, PLCB4, RHOB, AHNK, RHOA, ARHGEF6, LAT, ITPR3, ARHGEF2, ARHGEF3, RHOF, RALGDS
Role of tissue factor in cancer	3.21E+00	9.23E-02	VEGFA, IL8, CTGF, ARRB1, RRAS, ITGA6, IL1B, CSF2, CYR61, ITGB5, MMP1, ITGB3
Bile acid biosynthesis, neutral pathway	2.98E+00	6.90E-02	AKR1C1/AKR1C2, AKR1C3, AKR1C4, HSD3B7
VDR/RXR activation	2.66E+00	1.02E-01	IGFBP6, SPPI, MXD1, CDKN1A, HES1, CEBPB, THBD, CSF2, NCOA3
Glioma invasiveness signaling	2.30E+00	1.06E-01	RHOB, RRAS, RHOA, PLA2, RHOF, ITGB5, ITGB3
Methylglyoxal degradation III	2.17E+00	1.30E-01	AKR1C1/AKR1C2, AKR1C3, AKR1C4
Dopamine degradation	2.16E+00	1.05E-01	ALDH1B1, SULT1A1, SULT1A3/SULT1A4, SMOX
Androgen biosynthesis	2.06E+00	1.15E-01	AKR1C3, AKR1C4, HSD3B7
Reelin signaling in neurons	2.05E+00	9.41E-02	ARHGEF4, MAPT, ARHGEF6, ITGA6, ITGA5, ARHGEF2, ARHGEF3, ITGB3
IL17A signaling in fibroblasts	2.04E+00	1.25E-01	TRAF3IP2, NFKBIA, LCN2, CEBPB, MMP1
Estrogen-mediated S-phase entry	1.95E+00	1.43E-01	CDKN1A, E2F2, SKP2, CDC25A

**Figure 5.**

Exogenous GGPP rescues the antiproliferative effect of lovastatin. SKOV3 (A) and OVCAR5 (B) cells were incubated with GGPP (25  $\mu$ mol/L), FPP (25  $\mu$ mol/L), water-soluble cholesterol (400  $\mu$ g/mL), or CoQ10 (25  $\mu$ mol/L) alone or were coinubated with lovastatin (10  $\mu$ mol/L). To simplify the presentation, data from single-agent GGPP or FPP incubations are plotted separately in Supplementary Fig. S6. Data are presented as mean  $\pm$  SD ( $n = 3$ ). C and D, ovarian cancer cell lines, SKOV3 (C) and OVCAR5 (D), were transfected with siRNAs against key enzymes in the geranylgeranylation and squalene synthesis pathways, including PGGT1B, RABGGTB, and FDFT1. Control groups were transfected with nontargeting, medium GC siRNAs. Viable cells were measured at 24-hour intervals over a 120-hour period. Data are presented as the mean  $\pm$  SD ( $n = 3$ ).

reducing the amount of GGPP (geranylgeranyl pyrophosphate)—the isoprene lipid precursor of protein prenylation—by statins may compromise Rho/PLC signaling and suppress tumorigenesis.

The pleiotropic effects of statins on tumor suppression were further supported by our transcriptome analysis, which demonstrated that lovastatin-regulated genes participate in a wide spectrum of functional pathways including DNA replication, Rho/PLC signaling, and glycolysis, in addition to participating in cholesterol biosynthesis. The observed upregulation of mRNAs of mevalonate pathway genes such as HMG-CoA reductase is not surprising because negative feedback regulation of transcription in response to the inhibition of HMG-CoA reductase is well documented (33). Another potential antitumor mechanism of statins is suggested by our results demonstrating that statin treatment downregulated genes involved in DNA replication. Most notably, mRNA levels of seven MCM genes were significantly decreased by lovastatin treatment. MCM proteins are known to form replicative helicase complexes, which play a pivotal role not only in DNA initiation and elongation, but also in DNA damage response, transcriptional regulation, and modulation of chromatin structure (34, 35). Thus, statins may directly

or indirectly silence the expression of MCM genes, leading to cell-cycle arrest and accumulation of DNA damage.

Although the doses of lovastatin used in our chemopreventive mogp-Tag model and in the xenograft tumor model were well tolerated and were effective in suppressing tumor growth, they were higher than the doses used for treating hypercholesterolemia in patients. However, the doses used (25–45 mg/kg/day) in a phase I trial is similar to the doses used in this study (36). This prior report found that a dose of 25 mg/kg/day was well tolerated in the enrolled patients and resulted in delayed tumor growth in 1 patient with recurrent high-grade glioma (36). When administering with doses ranging between 25 and 45 mg/kg per day, some patients suffered from myotoxicity, the symptoms of which could be resolved by supplementation with CoQ10 (also called ubiquinone). In future clinical applications, it is most likely that statins will be applied as an adjuvant agent; hence, the dose in the combination setting is expected to be lower than the dose used as a single agent. Nevertheless, future studies are required to determine the maximum-tolerated dose in the combination regimen.

Using mouse models, we observed that lovastatin effectively inhibited tumor growth at both the precursor stage and in

established tumors, but had no noticeable effect on normal tissues. The reasons for the differential effects of statins on normal and neoplastic cells are unclear, but several possibilities can be postulated. First, ovarian tumor cells, as compared with normal cells, may have become more dependent on the mevalonate pathway for sustaining cellular survival and growth. In fact, mevalonate pathway activity is enhanced in many malignancies, including gastric, brain, and breast cancers among others (36–40). In addition, expression levels of HMG-CoA reductase are increased in neoplastic tissues (41, 42). Second, the transcriptional network has been reprogrammed in cancer cells, allowing the mevalonate pathway to control directly or indirectly transcriptional activities of key genes/pathways that collectively promote tumor progression. Although the precise mechanisms remain to be determined, statins may specifically suppress the transcriptional program of tumor cells and, subsequently, affect tumor growth and progression.

Our findings provide critical preclinical data and biologic rationales in evaluating lovastatin for prevention and treatment of ovarian cancer. STIC has been thought to be the precursor lesion in most ovarian HGSC (reviewed in ref. 43), and therapeutic intervention to surgically remove fallopian tubes that may harbor STICs or early cancers has been advocated for reducing ovarian cancer risk, especially for women with predisposing BRCA1/BRCA2 mutations. As a complement to this procedure, this study supports the need to further evaluate the clinical benefit of statins in preventing and treating this devastating disease.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Acknowledgments

The authors thank Ms. Emily Gerry, who assisted in preparing the article.

## Grant Support

This work was supported by DoD grants, W81XWH-11-2-0230 (to T.-L. Wang) and W81XWH-14-1-0221 (to K. Visvanathan/T.-L. Wang), and NIH grants, CA148826 (to T.-L. Wang), CA187512 (to T.-L. Wang), CA165807 (to I.-M. Shih), and Translational Research Network Program from Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan (to Y. Kobayashi).

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Received December 29, 2014; revised June 1, 2015; accepted June 8, 2015; published OnlineFirst June 24, 2015.



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# Clinical Cancer Research

## Mevalonate Pathway Antagonist Suppresses Formation of Serous Tubal Intraepithelial Carcinoma and Ovarian Carcinoma in Mouse Models

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*Clin Cancer Res* 2015;21:4652-4662. Published OnlineFirst June 24, 2015.

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